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### Permalink

<https://escholarship.org/uc/item/4q14s6b6>

### Journal

Cell, 111(4)

### ISSN

0092-8674

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### Publication Date

2002-11-01

### DOI

10.1016/s0092-8674(02)01040-1

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# Forward Transport: 14-3-3 Binding Overcomes Retention in Endoplasmic Reticulum by Dibasic Signals

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## Summary

Proteins with dibasic retention motifs are subject to retrograde transport to endoplasmic reticulum (ER) by COPI-coated vesicles. As forward transport requires escape from ER retention, general release mechanisms have been expected. Here, KCNK3 potassium channels are shown to bear two cytoplasmic trafficking motifs: an N-terminal dibasic site that binds  $\beta$ -COP to hold channels in ER and a C-terminal "release" site that binds the ubiquitous intracellular regulator 14-3-3 $\beta$  on a nonclassical motif in a phosphorylation-dependent fashion to suppress  $\beta$ -COP binding and allow forward transport. The strategy appears to be common. The major histocompatibility antigen class II-associated invariant chain lip35 exhibits dibasic retention, carries a release motif, and shows mutually exclusive binding of  $\beta$ -COP and 14-3-3 $\beta$  on adjacent N-terminal sites. Other retained proteins are demonstrated to carry functional 14-3-3 $\beta$  release motifs.

## Introduction

Newly synthesized proteins destined for membrane expression fold within the endoplasmic reticulum (ER) and travel forward through the *cis*-golgi complex (Ellgaard et al., 1999; Mellman and Warren, 2000). Misfolded or resident proteins that escape the ER are retrieved into COPI-coated vesicles for retrograde transport (Letourneur et al., 1994; Gaynor and Emr, 1997). COPI vesicles operate with notable specificity, recovering proteins through interaction with cytoplasmic dibasic "retrieval" motifs (Nilsson et al., 1989; Teasdale and Jackson, 1996; Zerangue et al., 2001). How COPI vesicular machinery (a complex of at least seven proteins) differentiates cargo for retention or forward transport is unknown. A masking strategy has been proposed in cases where expression is limited to fully assembled heteromeric complexes: the retention signal on one subunit hidden through assembly with its nonidentical partner(s) (Klausner, 1990; Zerangue et al., 1999; Standley et al., 2000; Zarei et al., 2001). Here, the cytoplasmic protein 14-3-3 $\beta$  is shown to act in a phosphorylation-dependent fashion to inhibit association of COPI proteins with dibasic sites and allow forward transport.

The seven known isoforms of 14-3-3 have been ascribed roles in biological activities as diverse as apoptotic cell death, cell cycle control, cell adhesion, mito-

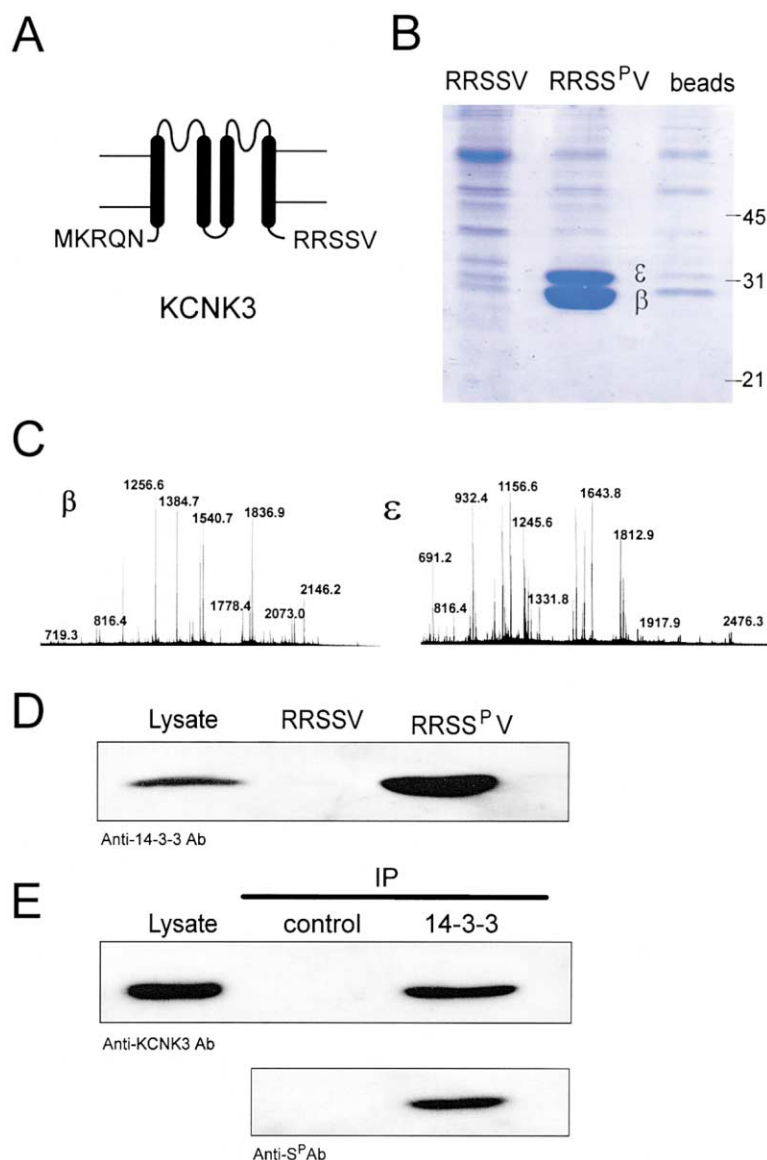
gen signaling, and neuronal plasticity (Fu et al., 2000; Tzivion and Avruch, 2002). These ~30 kDa, soluble, cytoplasmic proteins exert their recognized influences by binding to the consensus motifs -RSXS<sup>P</sup>XP- and -RXXS<sup>P</sup>XP- (hyphen indicates linkage to other residues) present on kinases, phosphatases, and transmembrane receptors after a serine in the motif is phosphorylated (S<sup>P</sup>) (Toker et al., 1990; Freed et al., 1994; Pallas et al., 1994; Muslin et al., 1996; Campbell et al., 1997; Craparo et al., 1997; Thorson et al., 1998; Zhou et al., 1999). A role for 14-3-3 in intracellular protein trafficking has been inferred from its capacity to rescue yeast cells with clathrin heavy chain mutations (Gelperin et al., 1995), enhance expression of neuronal acetylcholine receptors (Jeanclos et al., 2001), and alter levels of immune complexes on cells defective for clathrin-mediated endocytosis (Kuwana et al., 1998).

KCNK channels pass background potassium currents that regulate the behavior of nerves and muscles (Léage and Lazdunski, 2000; Goldstein et al., 2001). Subject to dynamic regulation in current magnitude, KCNK3 channels are homodimeric complexes (Lopes et al., 2001) implicated in responses of the heart and central nervous system to hormones, neurotransmitters, acidosis, hypoxia, and local and volatile anesthetics. In this study, surface expression of KCNK3 is found to be controlled by interplay of two sites: a dibasic site on the N terminus that binds  $\beta$ -COP to retain channels in ER and a C-terminal release site that binds 14-3-3 $\beta$  to a nonclassical motif (-RRSS<sup>P</sup>V), allowing forward transport. Thus, mutations that abolish 14-3-3 $\beta$  binding suppress trafficking to the plasma membrane while surface expression is restored by ablation of the dibasic site or restitution of 14-3-3 $\beta$  binding on the channel C terminus, for example, by creation of a classical 14-3-3 site as found in Raf1 kinase (-RSAS<sup>P</sup>EP). A mechanism is suggested by demonstration that (despite separation of retention and release motifs in the KCNK3 primary sequence) the channels bind  $\beta$ -COP or 14-3-3 $\beta$  in mutually exclusive fashion.

The presence of 14-3-3 $\beta$  binding motifs in other proteins subject to ER retention via dibasic signals suggests that operation of release sites may be common. Functional  $\beta$ -COP and 14-3-3 $\beta$  release motifs are demonstrated in three other membrane proteins. KCNK9 channels are shown to bear an N-terminal retention site identical to that in KCNK3 and a similar C-terminal release site (-RRKS<sup>P</sup>V). Nicotinic acetylcholine  $\alpha$ 4 subunits are shown to carry a dibasic motif analogous to a site that retains skeletal muscle  $\alpha$  subunits in ER (Keller et al., 2001) and a variant release site (-RSLSP<sup>P</sup>V-), both on a large cytoplasmic loop previously demonstrated to influence surface expression and to bind 14-3-3 $\beta$  (Jeanclos et al., 2001).

The cytoplasmic N terminus of the lip35 major histocompatibility antigen class II-associated invariant chain has a dibasic motif that maintains the subunit in ER (Schutze et al., 1994). Phosphorylation of the subunit has been shown to regulate forward trafficking of class II complexes (Anderson et al., 1999), alter release from

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**Figure 1. The KCNK3 C Terminus Is Phosphorylated and Binds 14-3-3 $\beta$  in Rat Brain**

(A) KCNK3 topology; N- and C-terminal residues shown.

(B) Homogenized and detergent-solubilized rat brain was incubated with immobilized RRSSV peptides, RRSS<sup>P</sup>V peptides, or peptide-free beads (as indicated). Interacting proteins were eluted, separated by SDS-PAGE, and stained with Coomassie blue. Two major products bound to the phosphorylated peptide (RRSS<sup>P</sup>V). Markers are kDa.

(C) MALDI analysis of major bound products from gel in (B) with key peaks labeled by size. Analysis using Sequest algorithms (NCBI) identify the proteins as 14-3-3 $\beta$  and 14-3-3 $\epsilon$  with a probability close to or equal to 1.

(D) Isolation of 14-3-3 $\beta$  from rat brain lysate by binding to phosphorylated KCNK3 peptide (RRSS<sup>P</sup>V) but not nonphosphorylated (RRSSV) peptide. Interacting proteins were eluted and separated by SDS-PAGE and visualized by Western blot analysis using a monoclonal antibody to 14-3-3 $\beta$  and ECL. Rat heart and skeletal muscle yield 14-3-3 $\beta$  by the same strategy (not shown).

(E) KCNK3 channels were copurified from rat brain when a monoclonal antibody was used to immunoprecipitate (IP) 14-3-3 $\beta$  (top). KCNK3 channels purified in this manner are visualized by antibodies to phosphoserine (bottom). Proteins separated by SDS-PAGE and visualized by Western blot analysis using a polyclonal anti-KCNK3 or anti-phosphoserine (S<sup>P</sup>) antibody, as indicated, and ECL. Control indicates immunoprecipitation with non-immune mouse IgG.

ER, and lead to 14-3-3 binding (Kuwana et al., 1998). Inspection reveals that lip35 carries a retention site (MHRRRSRSC-) adjacent to a variant release site (MHRRRSRSPC-). Here, we find that like KCNK3, lip35 binds either  $\beta$ -COP or 14-3-3 $\beta$  in mutually exclusive fashion. When the terminus is not phosphorylated,  $\beta$ -COP binds (even in the presence of 14-3-3 $\beta$ ); upon phosphorylation, 14-3-3 $\beta$  binds and  $\beta$ -COP association is suppressed.

A general mechanism for regulated anterograde trafficking is thus suggested. That 14-3-3 $\beta$  binds to KCNK3 to inhibit association of  $\beta$ -COP leading to release of homodimeric channels from ER retention, and that 14-3-3 $\beta$  binds to lip35 invariant chains to suppress  $\beta$ -COP binding under conditions that stimulate forward trafficking of heteromeric class II complexes (Anderson et al., 1999) indicates that forward transport of proteins with dibasic retention motifs is favored by phosphorylation-

dependent binding of 14-3-3 to the cargo, termination of COPI protein association, and release from ER retention.

## Results

### KCNK3 Peptide Identifies 14-3-3 $\beta$

KCNK3 subunits are predicted to have cytoplasmic N and C termini, two pore loops, and four transmembrane segments (Figure 1A; Goldstein et al., 2001). The five carboxy-terminal residues of KCNK3 (residues 390–394, -RRSSV) contain a consensus site for phosphorylation and suggest a potential PDZ-type binding motif. To probe the terminus for protein-protein interactions, a human heart cDNA library was screened by a yeast two-hybrid method using Gal4-based signaling and the last 16 residues of human KCNK3 as bait. Screening produced two independent isolates of the gene for 14-3-3 $\beta$  (GenBank accession number P31946) and the upstream

Table 1. Peptides Used in This Work

Description	Name in Text	Full Product
KCNK3 C terminus	-RRSSV	-CHSLSTFRGLMKRRSSV
KCNK3 C terminus + P	-RRSS <sup>p</sup> V	-CHSLSTFRGLMKRRSS <sup>p</sup> V
KCNK3 with Raf1 site	-RSASEP-	-CSTFRGLMKRSASEP
KCNK3 with Raf1 site + P	-RSAS <sup>p</sup> EP	-CSTFRGLMKRSAS <sup>p</sup> EP
KCNK3ΔV C terminus	-RSS	-CHSLSTFRGLMKRRSS
KCNK3ΔV C terminus + P	-RSS <sup>p</sup>	-CHSLSTFRGLMKRRSS <sup>p</sup>
KCNK3 N terminus	-KR-	MKRQNVAC-
NQ-KCNK3 N terminus	-NQ-	MNQNVAC-
KCNK9 C terminus	-RRKSV	-CFTDHQRLMKRRKSV
KCNK9 C terminus + P	-RRKS <sup>p</sup> V	-CFTDHQRLMKRRKS <sup>p</sup> V
Human α4 loop	-RSLSV-	-CAPGLAKARSLSVQHMSPPG
Human α4 loop + P	-RSLS <sup>p</sup> V-	-CAPGLAKARSLS <sup>p</sup> VQHMSPPG
Human α4 loop	-RR-	MPTWVRRVFLDIVPRC-
lip35 N terminus	MHRRRSRSC-	MHRRRSRSCREDQKC-
lip35 N terminus + P	MHRRRSRS <sup>p</sup> C-	MHRRRSRS <sup>p</sup> CREDQKC-
lip35 N terminus no S	MHRRRSREC-	MHRRRSRECREQKC-
HA peptide	HA peptide	YPYDVPDYA
FLAG peptide	FLAG peptide	DYKDDDDK

Peptides were synthesized as described in Experimental Procedures; a dash (-) indicates position of linkage to beads or other residues in the protein.

untranslated region of a known protein. This was unexpected, as the bait did not contain either common motif for 14-3-3 binding, -RSXS<sup>p</sup>XP- or -RXXS<sup>p</sup>XP- (Tzivion and Avruch, 2002).

#### KCNK3 Binds 14-3-3β in Rat Brain

Interaction of 14-3-3β and the KCNK3 bait was confirmed at the protein level with two KCNK3 peptides identical to the terminal 16 amino acids of the channel (Table 1). As 14-3-3 binding to classical motifs requires phosphorylation, the first peptide carried serine at the penultimate position (-RRSSV), the second a phosphoserine (-RRSS<sup>p</sup>V). The peptides were immobilized on beads and incubated with homogenized, detergent-solubilized rat brain. After washing to remove weakly associated proteins, bound products were eluted with 1% sodium dodecylsulfate (SDS) and separated by polyacrylamide electrophoresis (SDS-PAGE). Coomassie blue staining revealed that two major products bound only to the phosphorylated peptide (Figure 1B). These products were identified by MALDI-MS analyses to be 14-3-3β and 14-3-3ε (Figure 1C).

As expected from studies of classical motifs (Muslin et al., 1996), antibody staining demonstrated that native 14-3-3β from rat brain or heart or skeletal muscle bound only to the phosphorylated KCNK3 peptide (Figure 1D). Indicating association of native 14-3-3β and KCNK3 channels in vivo, the channels were copurified when a monoclonal antibody was used to immunoprecipitate 14-3-3β from rat brain (Figure 1E, top). The native KCNK3 subunits purified with 14-3-3 were phosphorylated on serine (Figure 1E, bottom); studies of cloned human KCNK3 channels (below) show that serine phosphorylation and 14-3-3 binding occur on the C-terminal motif of the intact channel.

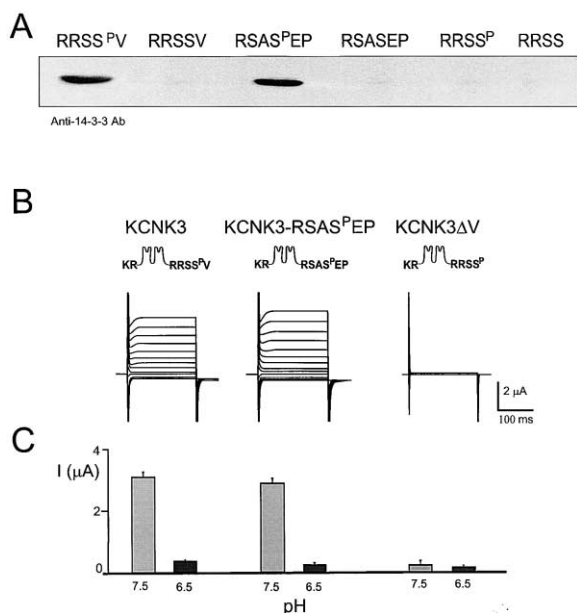
#### The Final Valine Is Required to Bind 14-3-3β

The 14-3-3 site in KCNK3 is like classical sites in requiring a phosphoserine but atypical both for lacking proline

(P) two positions downstream and in its location at the protein C terminus (Muslin et al., 1996; Yaffe et al., 1997; Rittinger et al., 1999). To evaluate these unique attributes, isolation of native 14-3-3β from rat brain was studied with various immobilized peptides. First, the KCNK3 residues -RRSS<sup>p</sup>V were replaced with a classical binding motif (-RSAS<sup>p</sup>EP-) that is present in the middle of the canonical 14-3-3 binding protein Raf1 kinase; this showed that, like the atypical site in KCNK3, a classical site could mediate phosphorylation-dependent binding of 14-3-3β when located at a C terminus (Figure 2A). Next, the KCNK3 site was shown to require its terminal valine since 14-3-3β did not bind to peptides terminating -RRSS or -RRSS<sup>p</sup> (Figure 2A).

#### KCNK3ΔV Channels Do Not Function

To assess the effect of 14-3-3β on KCNK3, function of channels expressed in *Xenopus laevis* oocytes was studied by two-electrode voltage clamp. Wild-type KCNK3 channels were first compared to mutants that terminated like the peptides that bound 14-3-3β via the classical motif found in Raf1 (KCNK3-RSAS<sup>p</sup>EP). The two channels produced currents that were indistinguishable (Figure 2B). Thus, cells expressing wild-type KCNK3 or KCNK3-RSAS<sup>p</sup>EP channels showed currents not seen in control cells and like those observed previously (Lopes et al., 2000, 2001). Currents produced by voltage steps showed a major component that appeared to activate and deactivate instantaneously and a smaller time-dependent component (Figure 2B). The channels also showed the same selectivity for potassium as judged by reversal potential (shifting 60mV ± 1mV and 60mV ± 2mV with a change in bath potassium from 5 to 100 mM for mutant and wild-type, respectively, n = 6–8 cells). Moreover, in both cases acidification of the external medium from pH 7.4 to 6.5 produced nearly complete blockade (Figure 2C). In contrast, mutant KCNK3 subunits that terminated like the peptide that could not bind 14-3-3β due to deletion of the final valine residue



**Figure 2. KCNK3ΔV Does Not Bind 14-3-3β or Pass Current**

(A) Isolation of 14-3-3β from rat brain was achieved by binding to the phosphorylated KCNK3 (RRSS<sup>PV</sup>) and Raf1 peptide (RSAS<sup>PEP</sup>) but not phosphorylated peptide without the final valine (RRSS<sup>P</sup>) or the nonphosphorylated peptides. Interacting proteins were eluted, separated, and visualized by Western blot analysis using a monoclonal antibody to 14-3-3β and ECL.

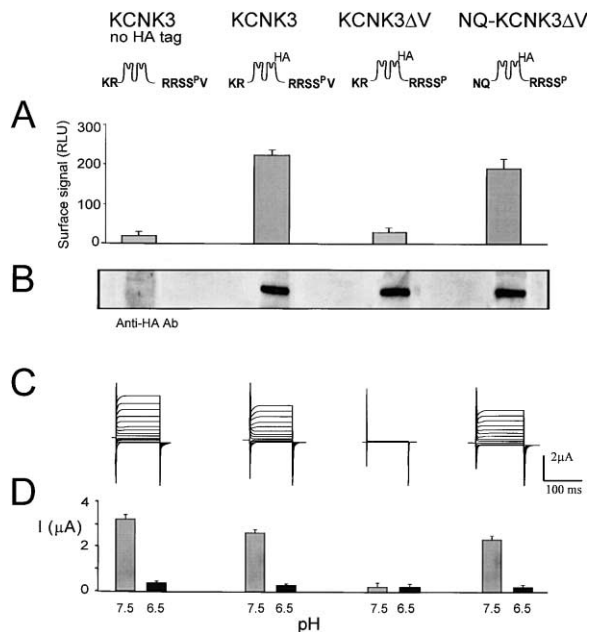
(B) Wild-type and KCNK3-RSAS<sup>PEP</sup> channels show similar current levels but KCNK3ΔV channels passed no current. Raw two-electrode voltage clamp recordings of oocytes expressing KCNK3 with various C termini as indicated. KCNK3 channels, KCNK3 channels with a Raf1-like C terminus (KCNK3-RSAS<sup>PEP</sup>), and KCNK3ΔV channels.

(C) Mean currents ( $\pm$  SEM) evoked in oocytes by step depolarization to +30mV for groups of 6–10 cells in external bath solutions of pH 7.4 and 6.5 as labeled.

(KCNK3ΔV) produced no currents in the oocytes (Figures 2B and 2C). Furthermore, a variant KCNK3 C-terminal peptide with diminished but not absent 14-3-3β binding (RRSS<sup>PV</sup>) was associated with mutant KCNK3 channels that showed depressed but not ablated current (KCNK3-RRSAV, KCNK3-RQSSV, and KCNK3-QRSSV, not shown). These findings suggested that 14-3-3 was required either to allow channels to reach the surface or to operate once inserted into the plasma membrane.

#### KCNK3ΔV Channels Do Not Reach the Surface

A binding assay demonstrated that KCNK3ΔV subunits fail to reach the plasma membrane. A hemagglutinin (HA) epitope tag was introduced into wild-type KCNK3 and KCNK3ΔV after the second pore domain, a site predicted to be exposed to the external milieu. Each oocyte was first studied by two-electrode voltage clamp, and then channel protein on the surface of intact cells was quantified using anti-HA antibody, a secondary antibody conjugated with horseradish peroxidase, and luminometry, a method developed by others (Zerangue et al., 1999). Whereas HA-tagged, wild-type KCNK3 subunits yielded a strong surface signal, HA-tagged KCNK3ΔV and untagged wild-type KCNK3 channels showed the same null level of fluorescence as uninjected oocytes (Figure 3A). Failure to detect KCNK3ΔV



**Figure 3. KCNK3ΔV Does Not Reach the Surface, whereas NQ-KCNK3ΔV Does**

(A) Surface expression of HA-tagged KCNK3, KCNK3ΔV, and NQ-KCNK3ΔV on single oocytes quantified by antibody labeling and luminometry. Plot is relative light units (RLU) for groups of 6–10 cells less nonspecific background fluorescence (mean  $\pm$  SEM). HA-tagged, wild-type KCNK3 subunits yielded a strong surface signal, while HA-tagged KCNK3ΔV and untagged wild-type KCNK3 channels showed the same null fluorescence of uninjected oocytes. NQ-KCNK3ΔV showed surface expression not significantly different from HA-tagged wild-type KCNK3.

(B) SDS-PAGE and Western blot analysis showed similar levels of HA-tagged KCNK3, KCNK3ΔV, and NQ-KCNK3ΔV in total cell lysates.

(C) Raw currents passed by single cells expressing indicated KCNK3 channels.

(D) Mean ( $\pm$  SEM) current from groups of 6–10 cells at pH 7.4 and 6.5.

channels on the surface was not due to failure to synthesize the protein; study of cells expressing HA-tagged wild-type and KCNK3ΔV channels by homogenization, SDS-PAGE, and Western blot analysis showed that similar levels of the two proteins were produced (Figure 3B). Since the biophysical attributes of HA-tagged KCNK3 channels were the same as their untagged counterparts, it seemed unlikely the tag further interfered with function of KCNK3ΔV channels (Figures 3C and 3D).

#### An N-Terminal Dibasic Motif Holds KCNK3ΔV Channels inside the Cells

KCNK3 subunits carry a potential ER retention motif, KR, at positions 2–3. To examine whether the motif had a role in failure of KCNK3ΔV subunits to reach the surface, the residues were altered by mutation to NQ (Figure 3A). The change restored function to KCNK3ΔV channels: NQ-KCNK3ΔV subunits bearing an HA-tag showed strong surface signals (Figure 3A), and although currents were smaller in magnitude, NQ-KCNK3ΔV channels were like untagged or HA-tagged wild-type KCNK3 channels in their response to voltage steps (Figure 3C), selectivity for potassium (shifting 58mV  $\pm$  2mV with a

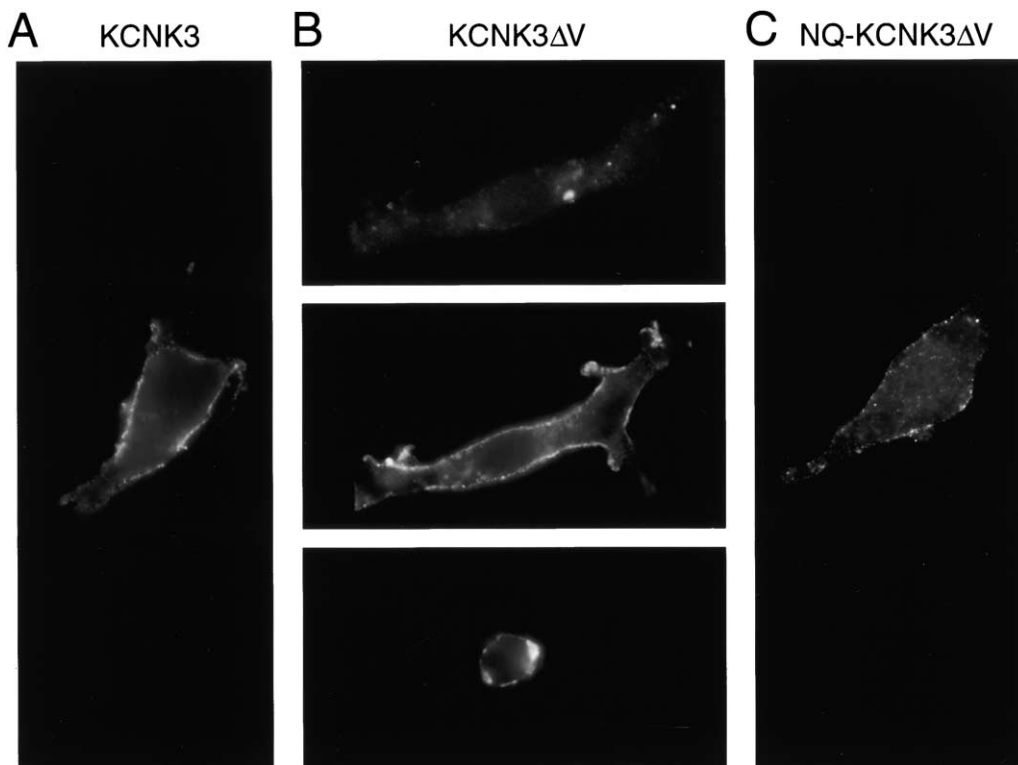


Figure 4. A Dibasic Motif Holds KCNK3 Channels in Endoplasmic Reticulum

COS-7 cells expressing HA and FLAG-tagged constructs of wild-type KCNK3, KCNK3 $\Delta$ V, or NQ-KCNK3 $\Delta$ V were labeled with anti-HA antibodies to stain the surface or anti-FLAG antibodies to visualize intracellular KCNK3 $\Delta$ V after permeabilization.

(A) Wild-type KCNK3 channels are observed at the plasma membrane.

(B) KCNK3 $\Delta$ V channels are not visualized on the plasma membrane (top), whereas transferrin receptors are visualized on the plasma membrane (middle). Permeabilization with detergent reveals KCNK3 $\Delta$ V channels accumulate in perinuclear areas (bottom).

(C) NQ-KCNK3 $\Delta$ V channels escape ER retention to reach the plasma membrane.

change in bath potassium from 5 to 100 mM,  $n = 6$  cells), and inhibition by external acidification (Figure 3D). This supported the conclusions that on its own the  $\Delta$ V mutation altered surface expression but not capacity to pass current and that 14-3-3 was not required for normal function of the channel once it had achieved surface expression.

KCNK3 channels expressed in mammalian tissue culture cells (COS-7) behaved like those studied in oocytes. Thus, wild-type channels were transported to the plasma membrane (Figure 4A); KCNK3 $\Delta$ V channels did not reach the surface (Figure 4B, top) while surface transferrin receptor was readily apparent (Figure 4B, middle); and finally, N-terminal mutation to form NQ-KCNK3 $\Delta$ V channels restored transport to the surface (Figure 4C). Cell permeabilization showed KCNK3 $\Delta$ V channels to be held in perinuclear areas as expected (Figure 4B, bottom). Immunostain and Western blot analyses revealed native 14-3-3 $\beta$  to be prominent in COS-7 cells (with a diffuse cytoplasmic pattern), suggesting why heterologous expression of recombinant 14-3-3 $\beta$  did not change the trafficking patterns of the three KCNK3 variants (not shown).

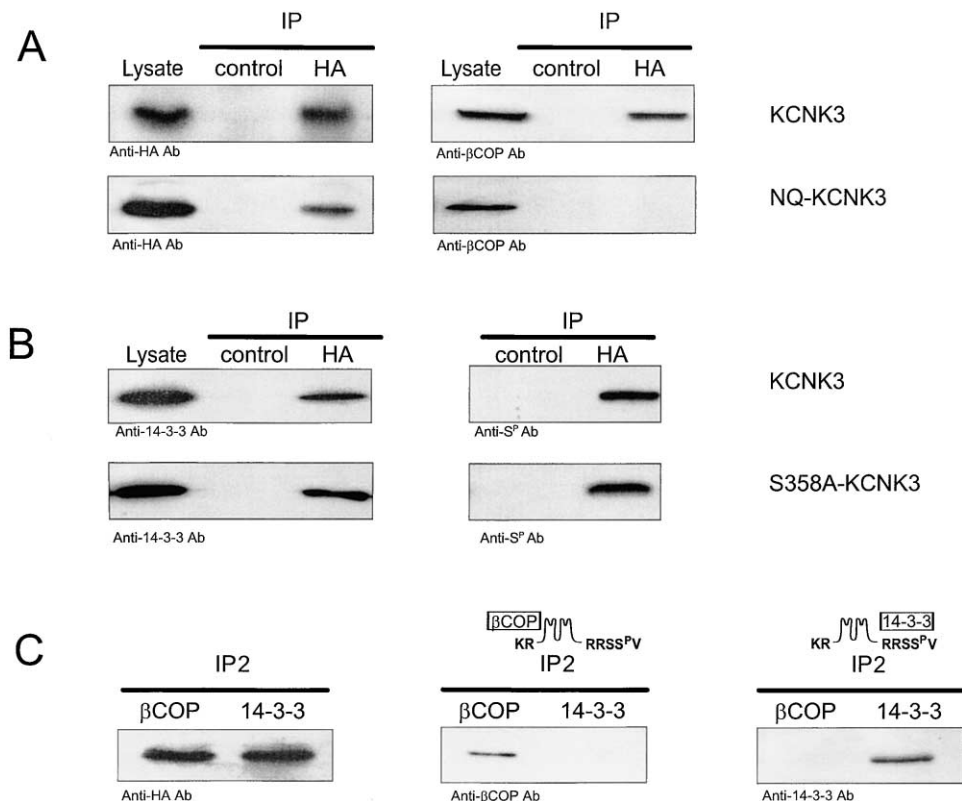
#### KCNK3 Binds Either $\beta$ -COP or 14-3-3 $\beta$

To confirm that ER retention of KCNK3 channels was mediated via the N-terminal dibasic signal and the coa-

tomer retrieval/retention mechanism (Ellgaard et al., 1999; Mellman and Warren, 2000), HA-tagged KCNK3 channels were expressed in COS-7 cells and associated native proteins evaluated by immunoprecipitation. Figure 5A shows that affinity purification with antibodies to HA isolated wild-type KCNK3 (top left) in association with  $\beta$ -COP (top right); conversely, KCNK3 channels altered from KR to NQ in the N-terminal dibasic motif were purified like wild-type (lower left) but were not associated with  $\beta$ -COP (lower right).

Immunoprecipitation of HA-tagged KCNK3 channels from COS-7 cells also led to isolation of associated native 14-3-3 $\beta$  (Figure 5B, top left). Like KCNK3 channels in rat brain (Figure 1E), the cloned channels associated with 14-3-3 carried phosphate on serine (Figure 5B, top right). Further evidence that phosphorylation and 14-3-3 binding occurred at the KCNK3 C terminus was offered by mutation of the clone to remove the only serine phosphorylation consensus site external to the C terminus (S358A KCNK3), as this did not interfere with 14-3-3 binding (Figure 5B, bottom left) or phosphoserine staining (Figure 5B, bottom right).

The HA immunoprecipitates were shown to contain two separable populations of KCNK3 subunits, one associated with  $\beta$ -COP, the other with 14-3-3 $\beta$ , as follows (Figure 5C): a second immunoprecipitation step (IP2) to reisolate  $\beta$ -COP led to repurification of KCNK3 (left) and



**Figure 5. KCNK3 Binds  $\beta$ -COP or 14-3-3 $\beta$  in Mutually Exclusive Fashion**

KCNK3 channels (HA-tagged) were expressed in COS-7 cells and studied by detergent extraction, immunoprecipitation, separation by SDS-PAGE, and visualization by Western blot analysis using antibodies as indicated with ECL.

(A) KCNK3 channels purified by immunoprecipitation (IP) with anti-HA antibodies and visualized with antibody to HA (top left) led to copurification of native  $\beta$ COP (top right). Conversely, NQ-KCNK3 channels mutated from KR to NQ in the dibasic motif were isolated (bottom left) in the absence of native  $\beta$ COP (bottom right). Control indicates immunoprecipitations with nonimmune mouse IgG.

(B) KCNK3 channels isolated as in (A) copurified native 14-3-3 $\beta$  (top left) that was phosphorylated on serine (top right). S358A KCNK3 channels mutated in the only internal consensus site for serine phosphorylation showed 14-3-3 binding (bottom left) and serine phosphorylation (bottom right) like wild-type. Control indicates immunoprecipitation with nonimmune mouse IgG.

(C) KCNK3 immunoprecipitates as in (A) were eluted from the beads with HA peptide and subjected to a second round of affinity purification (IP2) with antibody to  $\beta$ COP or 14-3-3 $\beta$  and visualized with antibody to HA (left),  $\beta$ COP (middle), and 14-3-3 $\beta$  (right); this revealed two populations of KCNK3 subunits: one with  $\beta$ COP, another with 14-3-3 $\beta$ ; failure to detect  $\beta$ COP and 14-3-3 $\beta$  indicate levels are at least 90% and 95% less than bound to an equal amount of KCNK3 in IP1 (Experimental Procedures).

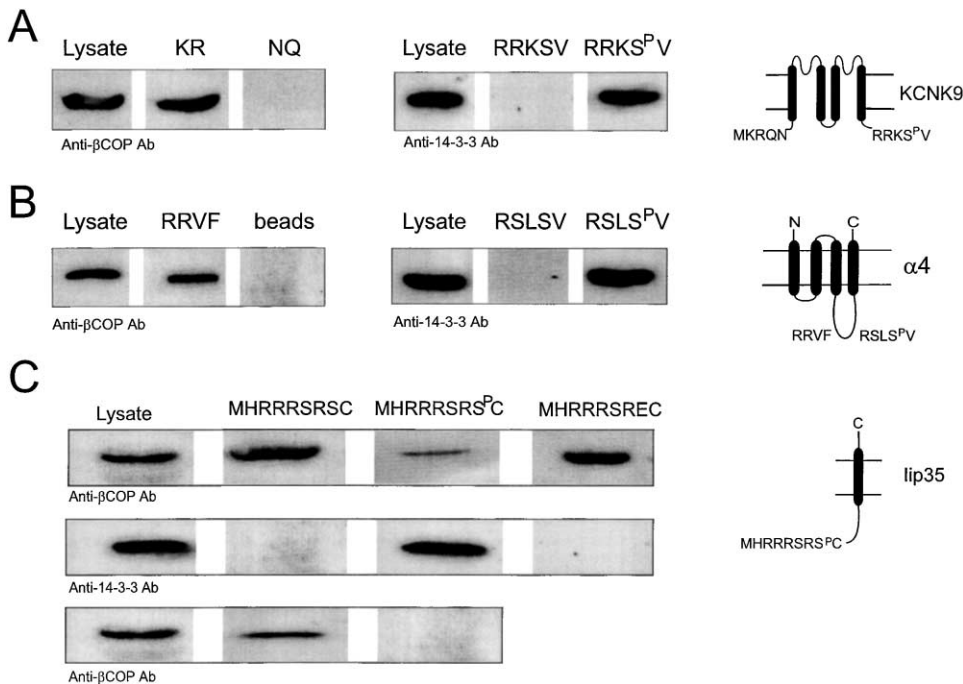
$\beta$ -COP (middle) but not 14-3-3 $\beta$  (right); conversely, IP2 to reisolate 14-3-3 $\beta$  yielded KCNK3 (left) and 14-3-3 $\beta$  (right) but not  $\beta$ -COP (middle). Failure to detect 14-3-3 associated with KCNK3 in the  $\beta$ -COP IP2 indicated its depletion to less than 5% of levels carried by unfractionated KCNK3 in IP1 (Experimental Procedures); depletion of  $\beta$ -COP in the 14-3-3 IP2 was similarly demonstrated to exceed 90%. These findings supported the conclusion that KCNK3 channels were held in ER via the COPI retention pathway and that phosphorylation-dependent binding of 14-3-3 suppressed COPI association to facilitate forward transport.

#### Release Motifs in KCNK9, $\alpha$ 4, and lip35

To explore the idea that proteins other than KCNK3 might employ a 14-3-3 release strategy, we studied peptides based on three plasma membrane subunits subject to ER retention by the COPI-dibasic signal pathway where prior studies had implicated phosphorylation and/or 14-3-3 in forward transport. KCNK9 channels are

54% identical to KCNK3 (the first nine residues of the two channels containing the KCNK3 dibasic retention motif are exactly alike) and predicted to have the same membrane topology (Kim et al., 2000). As expected, peptides matching the wild-type KCNK9/KCNK3 N terminus were found to bind  $\beta$ -COP from rat brain, whereas peptides that disrupted the motif (and allowed NQ-KCNK3 $\Delta$ V channels to escape ER retention) did not (Figure 6A, left). The C-terminal residues in KCNK9 subunits (-RRKSV) are similar to those in KCNK3 (-RRSSV), and the peptide analogous to the KCNK9 terminus was demonstrated, as anticipated, to bind 14-3-3 $\beta$  from rat brain when the penultimate serine was phosphorylated (Figure 6A, right).

Neuronal nicotinic acetylcholine receptors (nAChR) are ligand-gated, nonselective cation channels and attain surface expression after homo- or heteropentameric assembly (McGehee, 1999). Support for the operation of  $\beta$ -COP retention and 14-3-3 $\beta$  release motifs in neuronal  $\alpha$ 4 subunits was obtained as follows. Skeletal



**Figure 6. Retention and Release Motifs on KCNK9, α4, and lip35: Phosphorylated lip35 Binds 14-3-3β, Leading to Blockade of β-COP Association**

Isolation of βCOP and/or 14-3-3β from rat brain lysate using peptide-coupled beads. Interacting proteins were eluted, separated by SDS-PAGE, and visualized by Western blot analysis using monoclonal antibodies as indicated and ECL.

(A) The peptide analogous to the wild-type N terminus of KCNK9 (and KCNK3) binds β-COP from rat brain, while peptides with the NQ mutation do not (left). The C-terminal residues in KCNK9 subunits (RRKS<sup>P</sup>V) bind 14-3-3β from rat brain if the penultimate serine is phosphorylated (right). Drawing suggests the membrane topology of a KCNK9 subunit.

(B) A peptide analogous to a portion of the human neuronal α4 acetylcholine receptor large cytoplasmic loop binds β-COP from rat brain (-RR-, positions 347–348), left. A peptide identical to another portion of the loop binds native 14-3-3β when phosphorylated (-RSLSV<sup>P</sup>-, positions 464–468; GenBank accession U62433), right. Drawing suggests the membrane topology of an α4 subunit.

(C) Isolation of native β-COP from rat brain in the presence of native 14-3-3 (top): nonphosphorylated lip35 (and S<sup>P</sup> to E) peptides bind β-COP well but the peptide analogous to phosphorylated lip35 (MHRRRSRSC<sup>P</sup>) does not. Binding of native 14-3-3β is seen when the lip35 sequence is phosphorylated (MHRRRSRSC<sup>P</sup>) but not nonphosphorylated or the S<sup>P</sup> to E variant (middle). Prebinding of recombinant 14-3-3β ablates β-COP binding to the phosphorylated lip35 sequence (bottom). Drawing suggests the membrane topology of an lip35 subunit.

muscle α subunits are confined to ER by a dibasic signal, RK (positions 313–314) (Keller et al., 2001) and released after assembly of α, β, δ, and γ subunits (this appears to mask the α subunit retention signal). We found a similar motif in the large cytoplasmic loop of neuronal α4 subunits (RR, positions 347–348) that bound β-COP from rat brain (Figure 6B, left). Jeanclos and colleagues (Jeanclos et al., 2001) identified a motif in the same loop of α4 subunits (-RSLSVQ-, positions 438–443) that binds 14-3-3η, leading to an increase of homomeric α4 complexes on the surface (a smaller effect was seen on heteromeric α4β2 complexes). Consistent with their potential role as a KCNK3-like release site, these α4 residues were also found to bind 14-3-3β from rat brain when phosphorylated, -RSLSVQ- (Figure 6B, right).

II chains associate with newly synthesized class II molecules in ER to control their forward trafficking to antigen-processing compartments and prevent unwanted interaction of peptides with the class II binding groove during passage through the secretory pathway (Roche, 1990; Teyton, 1990; Cresswell, 1994). The operation of COPI retention and 14-3-3β release sites was strongly suggested by the observations that a dibasic motif retains lip35 in ER (Schutze et al., 1994) and that

phosphorylation regulates forward trafficking of class II complexes (Anderson et al., 1999) in association with 14-3-3 binding (Kuwana et al., 1998). Kuwana and co-workers (Kuwana et al., 1998) further showed that it is serine 8 of lip35 that bears phosphate in vivo and that mutation of the site to alanine suppressed 14-3-3 binding.

#### 14-3-3β Blocks β-COP Binding to lip35

The lip35 N terminus carries a basic retention motif, MHRRRSRSC-, followed by a suspected 14-3-3 release site. Using rat brain as a source of native β-COP and 14-3-3β, the nonphosphorylated terminus was found to bind a significant level of β-COP in the presence of 14-3-3β but not when the terminus was phosphorylated (MHRRRSRSC<sup>P</sup>-) (Figure 6C, top). Conversely, 14-3-3β bound only to the phosphorylated form of the N terminus (Figure 6C, middle). We suspected that poor binding of β-COP to the phosphorylated terminus was due to blocking of its binding site by 14-3-3β. To test this idea, β-COP binding was studied again using termini pre-treated with recombinant human 14-3-3β synthesized and purified from *E. coli*. As expected, exposure to 14-3-3β did not alter β-COP binding to the nonphos-



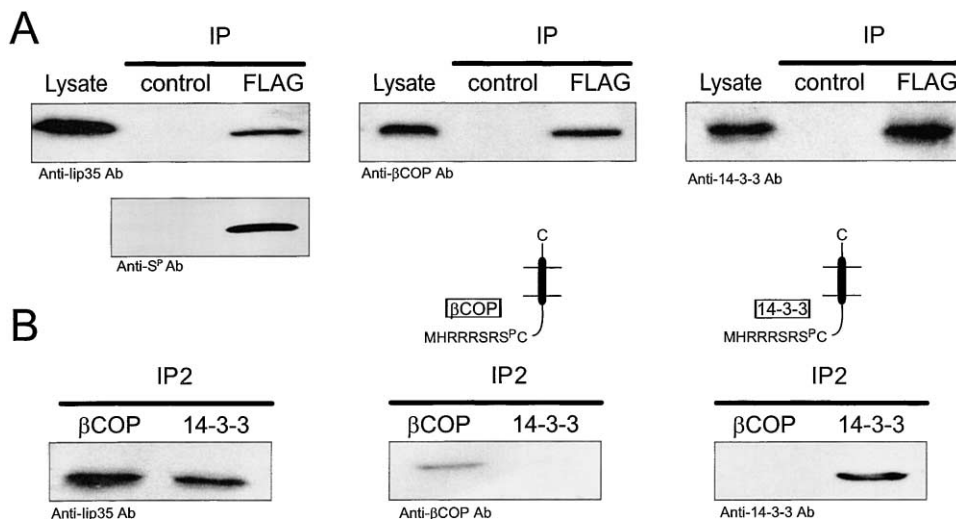


Figure 7. lip35 In Vivo Binds  $\beta$ -COP or 14-3-3 in Mutually Exclusive Fashion

COS-7 cells overexpressing lip35 (FLAG-tagged) were studied by detergent extraction, immunoprecipitation, SDS-PAGE, and Western blot analysis with ECL.

(A) lip35 was purified by immunoprecipitation with FLAG antibodies and visualized by antibody to lip35 (top left); anti-phosphoserine antibodies showed lip35 to be phosphorylated on serine (bottom left) as expected (Kuwana et al., 1998). Isolation of lip35 led to copurification of native  $\beta$ COP (middle) and native 14-3-3 $\beta$  (right). Control immunoprecipitation with nonimmune IgG.

(B) When the lip35 immunoprecipitate was eluted from the beads with FLAG peptide and subjected to a second round of affinity purification with antibody to  $\beta$ COP (left column) or 14-3-3 $\beta$  (right column) and visualized by antibody to lip35 (left),  $\beta$ COP (middle), and 14-3-3 $\beta$  (right), two populations of lip35 subunits were identified: one with  $\beta$ COP, another with 14-3-3 $\beta$ .

phorylated terminus but ablated binding to the phosphorylated form (Figure 6C, bottom). Supporting the conclusion that phosphorylation per se did not inhibit association of  $\beta$ -COP with its site, mutation of the S<sup>p</sup> site to a negatively charged glutamic acid (MHRRRSREC-) did not interfere with  $\beta$ -COP binding (Figure 6C, top) nor allow 14-3-3 $\beta$  binding (Figure 6C, middle). The retention and release sites thus operate in exclusive fashion such that 14-3-3 $\beta$  binding precludes  $\beta$ -COP association.

#### lip35 Binds Only $\beta$ -COP or 14-3-3 $\beta$ In Vivo

The behavior of lip35 in vivo supported operation of a mechanism for forward transport like that found for KCNK3: ER retention via the COPI-dibasic pathway and phosphorylation-dependent binding of 14-3-3 leading to inhibition of COPI binding and release from ER due to mutually exclusive binding. Thus, immunoprecipitation of lip35 (bearing a C-terminal FLAG tag) from COS-7 cells with FLAG antibodies yielded lip35 that was phosphorylated on serine (Figure 7A, left). As with KCNK3, the primary lip35 immunoprecipitate contained both native  $\beta$ COP and 14-3-3 $\beta$  (Figure 7A, middle and right), and the two mediators were found to reside in separate populations of lip35 complexes (Figure 7B): subjecting the immunoprecipitate to a second affinity purification step (IP2) to reisolate 14-3-3 $\beta$  yielded lip35 and 14-3-3 $\beta$  (left and right columns) but not  $\beta$ -COP (middle column), while reisolation of  $\beta$ -COP rendered lip35 and  $\beta$ -COP (left and middle columns) but not 14-3-3 $\beta$  (right column).

Although KCNK3 has multiple basic residues in its C-terminal 14-3-3 binding site (-KRRSSV), they do not appear to play a direct role in ER retention as do those at the lip35 N terminus. Thus, wild-type KCNK3 channels bind  $\beta$ -COP (Figure 5A, top) unless the N terminus is

mutated (Figure 5A, bottom), suggesting no other  $\beta$ -COP binding site is present. Similarly, N-terminal lip35 and KCNK3 peptides bind  $\beta$ -COP (Figures 6A and 6C) while C-terminal KCNK3 peptides do not (not shown). Finally, it is the wild-type N terminus that retains KCNK3 $\Delta$ V channels inside cells (Figures 2, 3, and 4), as its mutation to form NQ-KCNK3 $\Delta$ V channels allows release to the surface (Figures 3 and 4).

#### Discussion

Regulated surface expression of plasma membrane receptors is key to normal cellular physiology (Elgaard et al., 1999; Mellman and Warren, 2000). Membrane trafficking proceeds under tight control to establish steady-state expression levels, respond to acute stimuli, and monitor the quality of secreted multimeric products. One cellular strategy is to maintain proteins in ER through retention and recovery in COPI-coated vesicles via "dibasic" recognition of sites of one, two, or three sequential arginine or lysine residues (Nilsson et al., 1989; Teasdale and Jackson, 1996; Zerangue et al., 2001). While masking of retention signals appears to allow some mixed complexes to escape the ER (Zerangue et al., 1999; Standley et al., 2000; Zarei et al., 2001), general methods to overcome suppression have been unknown. In this study, we show that surface expression of KCNK3 channels is achieved by phosphorylation-dependent binding of 14-3-3 $\beta$  and release from COPI-mediated ER retention. The mechanistic basis for forward transport of KCNK3 appears to be mutually exclusive binding of  $\beta$ -COP and 14-3-3 $\beta$ . Functional  $\beta$ -COP retention and 14-3-3 $\beta$  release motifs are demonstrated in three other proteins (KCNK9 channels,  $\alpha$ 4 nicotinic acetylcholine

receptors, and lip35). Retention and release sites on the immune system protein are adjacent and 14-3-3 $\beta$  binding (rather than motif phosphorylation) is demonstrated to suppress  $\beta$ -COP binding. The findings show 14-3-3 to be a generic mediator of release from ER retention, expand recognized binding motifs for 14-3-3, and suggest a mechanism for regulating forward trafficking of homomeric and heteromeric protein complexes.

#### Trafficking to the Plasma Membrane

KCNK channels show widespread tissue expression from at least 17 separate genes (Lesage and Lazdunski, 2000; Goldstein et al., 2001). Subject to regulation in magnitude and character by a panoply of natural and medicinal agents, the channels are notable in both structure and function because they carry two pore-forming P loops on each subunit (K<sub>2P</sub> channels) and form background potassium channels (Ketchum et al., 1995; Goldstein et al., 1996; Bockenhauer et al., 2001; Ilan and Goldstein, 2001; Zilberberg et al., 2001). Background channels regulate the function of nerves and muscles because increasing potassium leak stabilizes cells at negative resting potentials, thereby diminishing excitability. Slow changes in KCNK3 current magnitude have been implicated in cardiac and nervous tissue responses to hormones, neurotransmitters, and drugs—some of which operate through cAMP-mediated pathways (Kim et al., 1999; Kindler et al., 1999; Patel et al., 1999; Czirjak et al., 2000; Lopes et al., 2000; Talley et al., 2000). As the 14-3-3 $\beta$  release site in KCNK3 is a canonical cAMP-dependent protein kinase A (PKA) phosphorylation site, regulated expression of KCNK3 channels may be a pathway for altering leak current magnitude in response to external stimuli. Indeed, activity of potassium channels already in the membrane can also be altered by 14-3-3 in a PKA-dependent fashion in the case of a human cardiac channel (Kagan et al., 2002) and in a calcium/calmodulin kinase II-dependent manner (via a third protein) in *Drosophila* neuromuscular junctions (Schopperle et al., 1998; Zhou et al., 1999).

Nicotinic receptors are neurotransmitter-gated ion channels implicated in memory, nociception, addiction, epilepsy, and Parkinson's disease (Lindstrom, 1997). The receptors assemble from 11 subunit isoforms,  $\alpha$ 2–9 and  $\beta$ 2–4 (Drisdell, 2000), emerging on the surface as  $\alpha$  subunit homopentamers or  $\alpha\beta$  heteropentamers (McGehee, 1999). Keller and colleagues (Keller et al., 2001) showed that a dibasic motif in the large cytoplasmic loop of skeletal muscle  $\alpha$  subunits mediates ER retention, while Jeanclos and coworkers (Jeanclos et al., 2001) implicated a 14-3-3 protein (native isoform undetermined) in forward transport of nicotinic receptors, demonstrating that activation of PKA by forskolin enhanced binding of recombinant 14-3-3 $\eta$  to the  $\alpha$ 4 loop sequence -RSLSPVQ- and increased surface expression of  $\alpha$ 4 channels over 5-fold and  $\alpha$ 4 $\beta$ 2 receptors ~20%; others observed a 2-fold increase in  $\alpha$ 4 $\beta$ 2 receptor surface expression under similar conditions (Gopalakrishnan, 1997). The findings here that an  $\alpha$ 4 cytoplasmic loop binds  $\beta$ -COP and shows phosphorylation-dependent interaction with 14-3-3 $\beta$  supports the notion that escape of  $\alpha$ 4 homomers from ER results from 14-3-3 bind-

ing, whereas  $\alpha$ 4 $\beta$ 2 receptors (thought to be the prominent *in vivo* species) can employ  $\beta$ 2 masking and are thus less sensitive to 14-3-3.

#### lip35 Invariant Chains

Invariant chains play a critical role in trafficking of newly synthesized class II complexes to antigen-processing compartments. lip35 is retained in ER by a dibasic motif (Schutze et al., 1994) and enjoys anterograde transport in a phosphorylation (Anderson et al., 1999) and 14-3-3-dependent fashion (Kuwana et al., 1998). Kuwana and coworkers (1998) suggested that regulation of 14-3-3 binding (via phosphorylation) could thus serve to control immune signal transduction. These observations can be rationalized by mutually exclusive binding of  $\beta$ -COP and 14-3-3 $\beta$  as observed here: lip35 binds  $\beta$ -COP and is retained in ER when the N terminus bears no phosphate (MHRRRSRSC-), while phosphorylation of the adjacent release site (MHRRRSRSPC-) leads to 14-3-3 binding, suppression of  $\beta$ -COP association, and forward transport.

#### A New Role and a Wealth of Nonclassical Sites for 14-3-3

This study supports the conclusion that release from ER retention should be added to the long list of functions attributed to 14-3-3 variants. Previously implicated in trafficking by studies of endocytosis-deficient yeast cells (Gelperin et al., 1995) and neuronal acetylcholine receptors (Jeanclos et al., 2001), it was Kuwana and colleagues (Kuwana et al., 1998) who explicitly suggested that 14-3-3 might serve to regulate forward transport of lip35. How broadly operative this pathway is remains to be assessed. Just as COPI-mediated retention/retrieval motifs are widespread and variable in sequence, so too, the four nonclassical 14-3-3 binding sites identified here are diverse enough to suggest that many serine kinase phosphorylation sites will prove capable of 14-3-3 binding (KCNK3, -RRSSV; KCNK9, -RRKSV;  $\alpha$ 4, -RSLSV-; and lip35, -RSRSC-). Further, it is possible that some classical sites mediate release, as altering the KCNK3 C terminus to a classical motif yielded both 14-3-3 binding and surface expression (Figure 2). It is also unknown whether 14-3-3-mediated release from ER is limited to dibasic sites on membrane proteins, can operate with other retention motifs such as KDEL (Pelham, 1990), or employs additional proteins to proceed.

#### Mutually Exclusive Binding and Release from ER Retention

Regulated release of correctly assembled mixed complexes from ER via masking of retention signals is a recognized strategy to control the type and quality of membrane proteins that reach the surface (Klausner, 1990; Zerangue et al., 1999; Standley et al., 2000; Zarei et al., 2001). Here, we demonstrate that 14-3-3 can serve as a general mediator for forward transport through inhibition of COPI protein binding, allowing release from ER retention. Evidence for mutually exclusive binding is given in one case where retention and release sites are separate in the primary sequence (KCNK3) and another where they are adjacent (lip35). While steric interference

can rationalize binding of either  $\beta$ -COP or 14-3-3 on adjacent lip35 sites, it is not known if the regulators interact directly when the sites are separated. Further support for the operation of this pathway *in vivo* are the presence of 14-3-3 and PKA in ER and golgi (Nigam, 1989; Kuwana et al., 1998), the effects of kinase activators on 14-3-3 binding and forward transport of  $\alpha 4$  and  $\alpha 4\beta 2$  receptors (Gopalakrishnan, 1997; Jeanclos et al., 2001), and *cis*-acting mutations that leave dibasic signals intact yet act on the separate site to produce forward transport of Emp47p (Schroder-Kohne, 1998). Evidence that 14-3-3 augments forward transport not only of homomeric KCNK3 channels and  $\alpha 4$  receptors but heteromeric complexes (such as  $\alpha 4\beta 2$  receptors and class II-invariant chain complexes) where intersubunit retention motif masking has been suspected to be sufficient suggests 14-3-3 may serve a role in normal release from ER in both cases. As many proteins carry both ER retention and 14-3-3 binding motifs, the mechanism described may operate broadly.

## Experimental Procedures

### Yeast Two-Hybrid Identification of 14-3-3 $\beta$

The Invitrogen ProQuest system, a human heart library, and the pDBLeu vector encoding the last 16 residues in human KCNK3 as bait were employed. One million clones were screened and the inserts in three resultant clones were isolated, sequenced, and identified as described in Results.

### Mutagenesis

hKCNK3 was cloned from a human brain cDNA library (Clontech, Palo Alto, CA) with the primers 5'-CGATGAAGCGGAGAACGTGCGCAC-3' and 5'-GGGCAGAAAGCAGCAGGGGCAGTC-3' and subcloned into pRAT (Bockenhauer et al., 2001). Mutations were made with an XL Quickchange kit (Stratagene, La Jolla, CA) and confirmed by DNA sequencing. FLAG tags (DYKDDDDK) were inserted before the start methionine; HA tags (YPYDVPDYA) were inserted at position 213 in KCNK3.

### Antibodies

Purchased polyclonal (p) and monoclonal (m) antibodies used included: KCNK3 (p, APC024, Alomone Labs, Jerusalem, Israel); 14-3-3 $\beta$  (m, SC1657, Santa Cruz Biotechnology, Santa Cruz, CA);  $\beta$ -COP (m, PC175, Calbiochem, LaJolla, CA);  $\beta$ -COP (p, PA1601, Affinity Bioreagent, Golden, CO); Phosphoserine (p, AB1603, Chemicon International, Temecula, CA); FLAG (m, F3165, Sigma-Aldrich, St. Louis, MO); HA tag (m, 1583816; m, 1867423 and p, 1867423, Roche Molecular Biochemicals, Indianapolis, IN); Transferrin receptor (p, SC9099, Santa Cruz, Santa Cruz, CA); Protein disulfide isomerase (PDI) (p, SPA890, StressGen Biotech., Victoria, Canada); and lip35 (m, BYA91341, Accurate Chemicals, Westbury, NY).

### Peptide Binding Studies

Peptides and phosphopeptides were synthesized by the W.M. Keck Biotechnology Resource Center (New Haven, CT) with the exceptions of HA (I2149) and FLAG (F3290), which were purchased from Sigma. A cysteine residue was added to the terminus of each peptide that was linked to Sulfolink Coupling Gel (Pierce, Rockford, IL), according to manufacturer's instructions. Rat brain extract was prepared by homogenizing two frozen rat brains in 20 ml of cold Buffer A (10 mM HEPES [pH 7.4], 150 mM NaCl, protease inhibitor cocktail tablets [PI; Roche Molecular Biochemicals Indianapolis, IN]) and centrifuged at  $800 \times g$  for 10 min at 4°C. The resulting supernatant was solubilized with 1% Triton X-100 for 1 hr and centrifuged for 1 hr at  $100,000 \times g$  at 4°C. The final supernatant was then passed over the various peptides immobilized on Sulfolink Gel (2 mg protein/ml of coupled gel). The gel was washed with 6 bed volumes of phosphate buffered saline (PBS) with 1% Triton X-100 and bound proteins were eluted with SDS-PAGE sample buffer and analyzed

by Western blotting and MALDI-MS (Keck Center). Extracts of rat heart and skeletal muscle were prepared similarly. Binding assays with recombinant 14-3-3 proteins were supplemented with 1% bovine serum albumin.

### Immunoprecipitation Protocols

Proteins were extracted from rat brains by homogenization and incubation on ice for 1 hr in cold Buffer B (50 mM Tris [pH 8.0], 1 mM EDTA, 150 mM NaCl, 1% NP-40) plus 0.5% deoxycholate and 0.1% SDS followed by centrifugation at  $13,000 \times g$  for 30 min at 4°C. The resulting supernatant (7 mg/ml total protein) was clarified by centrifugation at  $50,000 \times g$  for 30 min and dialyzed for 12 hr against Buffer B with 1% Triton X-100 at 4°C. Triton X-100 was added to 1% before extracts were incubated with 5  $\mu$ g of 14-3-3 monoclonal antibody or control mouse IgG (Sigma) overnight at 4°C. Immune complexes were isolated with 50  $\mu$ l of Protein G Sepharose (Amersham Pharmacia Biotech, San Francisco, CA), washed with Buffer B with 1% Triton X-100, and eluted with SDS-PAGE sample buffer. SDS-PAGE (12%) and Western blotting were performed by standard methods using ECL (Pierce, Rockford, IL). For protein expression analysis in oocytes, cells were homogenized, subjected to SDS-PAGE, and visualized by Western blot analysis.

Two-step affinity purification of HA-tagged KCNK3 or FLAG-tagged lip35 from COS-7 cells was performed 24 hr after transfection using Lipofectamine (Invitrogen, Carlsbad, CA) by incubation for 1 hr in Buffer C (50 mM Tris [pH 8.5], 100 mM NaCl) with 1% Triton X-100 and 0.1% SDS (HA) or 1% CHAPS (FLAG) and centrifugation at  $13,000 \times g$  for 20 min at 4°C. Extracts (2.5 mg total protein) were incubated with 5  $\mu$ g HA or FLAG monoclonal antibody or control mouse IgG overnight at 4°C. Immune complexes were isolated with 50  $\mu$ l of Protein G Sepharose, washed with Buffer C plus 1% Triton X-100 (HA) or 1% CHAPS (FLAG), and eluted from the beads with 50  $\mu$ M HA or FLAG peptide. Second-round immunoprecipitations (IP2) were performed on 0.1 mg of recovered protein by incubation with antibodies to  $\beta$ -COP or 14-3-3 $\beta$ , isolation via Protein G, and elution with SDS-PAGE sample buffer. One-fiftieth of the unfractionated extract ( $\sim 50 \mu$ g) was loaded in the control lysate lane of each gel. To provide a conservative estimate for the minimal detectable level of 14-3-3 or  $\beta$ -COP protein associated with KCNK3 in IP1 (and thereby assess the relative depletion of each in IP2), the amount of KCNK3 in an IP2 was normalized to its level in IP1 by serial dilution using Western blot analysis. The degree of further IP1 dilution that still allowed for specific visualization was more than 1/20 for 14-3-3 and 1/10 for  $\beta$ -COP; this indicated that 5% and 10% of control levels of 14-3-3 and  $\beta$ -COP were readily observed and depletion was greater than 95% and 90%, respectively.

### Production of Recombinant 14-3-3 $\beta$

The gene encoding human 14-3-3 $\beta$  (P31946) was cloned via the two-hybrid screening of the human heart library and subcloned into pGEX 6P-1; 14-3-3 $\beta$  was released from the glutathione S-transferase (GST) fusion protein with PreScission™ Protease (Amersham Pharmacia Biotech).

### Immunofluorescence

COS-7 cells were seeded on poly-L-lysine-coated coverslips, transfected with HA or Flag-tagged KCNK3 using calcium phosphate, and 48 hr later were fixed in 4% paraformaldehyde for 30 min at 4°C. For surface staining, cells were blocked with 20 mM sodium phosphate (pH 7.4), 10% goat serum, 400 mM NaCl for 1 hr at 4°C, incubated with a rat monoclonal anti-HA antibody for 1 hr, washed, and stained with Oregon Green-conjugated anti-rat antibody (Molecular Probes). Cells were permeabilized by including Triton X-100 (0.3%) in the blocking step, followed by incubation with a mouse monoclonal anti-FLAG antibody and visualized with Oregon Green-conjugated goat anti-mouse antibody. Plasma membrane or ER staining was confirmed by double labeling with polyclonal antibodies to transferrin receptor or PDI, respectively, and Texas-red conjugated goat-anti-rabbit antibodies.

### Electrophysiology

Oocytes were isolated from *Xenopus laevis* frogs (Nasco, Atkinson, WI) treated with collagenase to ease removal of the follicular layer

and injected with 0.1–1 ng of KCNK3 cRNA in 46 nl of sterile water. Currents were measured 48 hr after injection by two-electrode voltage clamp (Warner Instruments Corp., Hamden, CT). Data were filtered at 1 kHz and sampled at 4 kHz. Electrodes of 1.5 mm borosilicate glass tubes (Garner Glass Co., Claremont, CA) contained 3 M KCl and had resistances of 0.3 to 1 mOhm. Recordings were performed at room temperature with perfusion of 0.4–1 ml/min ND96 (in mM) 93 NaCl, 5 KCl, 1 MgCl<sub>2</sub>, 0.3 CaCl<sub>2</sub>, 5 HEPES, pH was adjusted to 7.5 or 6.5 where appropriate with NaOH or HCl. Potassium in the bath was altered by isotonic substitution of KCl for NaCl. Holding potential in all cases was –80 mV. Currents were evoked by step depolarization from –135 mV to +60 mV in 15 mV increments. Acid sensitivity was determined by exchanging external bath solutions during repeated step depolarization from holding potential to +30 mV at 10 s intervals.

#### Surface Binding Assay for KCNK3

*Xenopus* oocytes were prepared and injected as described for electrophysiology. The assay was as described by Zerangue and co-workers (Zerangue et al., 1999). Briefly, oocytes were blocked for 30 min in ND96 with 1% bovine serum albumin (BSA) at 4°C, treated with 5 µg/ml mouse monoclonal anti-HA antibody in 1% BSA for 60 min at 4°C, washed, and incubated with HRP-conjugated secondary antibody (goat anti-mouse IgG) in 1% BSA for 60 min at 4°C. Cells were washed (1% BSA, 4°C, 60 min) and transferred to ND96 without BSA. Individual oocytes were placed in 50 ml SuperSignal ELISA Femto Maximum Sensitivity substrate (Pierce, Rockford, IL) at 22°C for 1 min. Chemiluminescence was quantified with a MultiSkan ELISA plate reader.

#### Acknowledgments

This work was supported by grants to S.A.N.G. from the NIH. We are grateful to M.J. Caplan, the anonymous reviewers, and the Editor for constructive critical feedback, R. Goldstein for assistance with graphics, and J. Crawford and T. Abbott for excellence in support of peptide synthesis and MALDI analyses.

Received: March 13, 2002

Revised: September 10, 2002

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